PHARMACOLOGY NO. 884

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

COMMITTEE:
Dr. Cattell
Dr. Gardner
Dr. Jacobson

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grants

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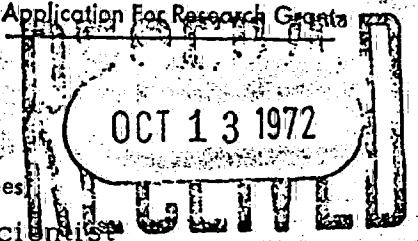

Date:

1. Name of Investigator(s): (include Title and Degrees)
Albert Castro, Ph.D., Senior Scientist

2. Institution & Address:
Papanicolaou Cancer Research Institute
1155 N.W. 14th Street
Miami, Florida 33136

3. Short Title of Project: NICOTINE IN BLOOD: DETECTION BY RADIOIMMUNOASSAY

4. Proposed Starting Date: February 1, 1973

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Descripton of Objectives or Specific Aims:

The major objective of this proposal is to develop means of quantifying nicotine and its major metabolites in blood at the levels achieved as a consequence of tobacco smoking. In view of the low levels of substances to be measured and in view of our experience in detecting hormones and drugs, we believe that radioimmunoassay is the procedure of choice. The research proposal covers the development of specific antisera against nicotine, cotinine, hydroxycotinine and desmethylcotinine. As antisera become available, protocols will be developed for assaying nicotine and its metabolites using "wet" chemistry techniques. However, we think that standard radioimmunoassay methodology can be simplified significantly by converting to "solid-phase." Specifically, as "wet" chemistry protocols are developed and field-tested, we will couple specific antisera to solid supports (e.g. silanized controlled pore glass), and solid-phase protocols will be developed and tested in parallel. Our preliminary experience with solid-phase radioimmunoassays of other substances (e.g. thyrotropin and progesterone) indicate that the time required for a given assay may be reduced seventy-fold.

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7. Give a Brief Statement of your Working Hypothesis: Appended

Source: https://www.industrydocuments.ucsf.edu/docs/...

8. Details of Experimental Design and Procedures: (Attach Separate Pages) Appended

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)
Appended

10. Additional Requirements:

Nuclear of Chicago 300-place, B-channel liquid scintillation counter

We plan to use ^{3}H-nicotine as the displacement tracer. Therefore liquid scintillation counting is essential to the proposed research project.

Animals: The projected field tests require large numbers of animals. Possibly these costs could be reduced if we could gain access to blood samples used in other field tests supported by CTR.

11. Biographical sketches of all principal and professional personnel (append) Appended

12. List of publications: (Five most recent as pertinent) (append) Appended

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13. Budget: (1st year)

A. Salaries (Personnel by names)	% time	Amount
Professional		
Albert Castro, Ph.D., Principal Investigator	30%	9,000
Alfred Chung, M.S., Organic Chemist	50%	7,000
Fringe Benefits (10% of salary) Includes FICA medical insurance, life insurance and unemployment		1,600
Technical		
Laboratory Technician	100%	6,000
Fringe Benefits (10% of salary)		600
	Sub-Total	24,200
B. Consumable Supplies (list by categories)		
Animals: 100 rabbits @$7		700
maintenance $0.30/day/rabbit, average 30 days		900
Chemicals and glassware		600
Radioactive isotopes		1,400
	Sub-Total	3,600
C. Other Expenses (itemize)		
Travel to Scientific Meetings, 1 trip		500
	Sub-Total	500
D. Permanent Equipment (itemize)		
Nuclear of Chicago liquid scintillation counter 300-place, 3-channel		16,000
		16,000
E. Overhead (15% of A+B+C)		4,245
	Total	48,545

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	26,015	3,780	500	-0-	4,544	34,839
Year 3	27,966	3,969	500		4,865	37,300

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature _____
Director of Project
503/666-7959
Telephone

Signature _____
Business Officer of the Institution
305/371-5572 Telephone

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Source: https://www.industrydocuments.ucsf.edu/docs/nry10000

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Non-renal renin-angiotensin systems in man	Oregon Heart Association	$9,100	July 1971-July 1973
Studies of Renin-angio-aldosterone inter-relationship in acute saline depletion	Oregon Heart Association	10,376	July 1971-July 1972
Renin angiotensin system	Commack Foundation	7,800	July 1971-July 1972
Insulin release	Oregon Diabetes Association	2,000	July 1971-July 1972

ending

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Source: https://www.industrydocuments.ucsf.edu/docs/nryl0000

7. Working Hypothesis

Depending on the dose and the route of administration, nicotine may stimulate or depress sympathetic and parasympathetic ganglion cells, the adrenal medulla, motor end plates of skeletal muscle, ciliated epithelial cells of the airways and chemoreceptors of the coronary, pulmonary, aortic and carotid arteries (for review see Comroe, 1960). This listing of pharmacologic effects is by no means exhaustive. Still the point remains that it is not known which if any of these pharmacologic effects play a role in the disease processes statistically linked with the use of tobacco and possibly with nicotine.

The basic problem is that little is known of the concentrations of nicotine and its metabolites achieved in blood, other body fluids and tissues as a consequence of smoking. We believe that modern assay technology can remedy this situation, and we therefore propose to develop radioimmunoassays for nicotinine, cotinine, hydroxycotinine and desmethylcotinine. Emphasis will be placed on simplicity and rapidity of use as well as on specificity and sensitivity. We then plan to make these assays available to all interested investigators to permit a direct reexamination of the actions of nicotinine and its metabolites as a function of inhalation of tobacco smoke.

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Source: https://www.industrydocuments.ucsf.edu/docs/mnjl0000

Background

As pointed out by Isaac and Rand (1969), nicotine is one of the most commonly used drugs. Furthermore, few drugs are administered in as complex a manner as is nicotine in tobacco smoke: generally eight or more small doses are taken by inhalation at roughly one minute intervals in the smoking of one cigarette, and the "addicted" smoker repeats this process at 15-30 minute intervals throughout the waking day.

The fate of the dose of nicotine in tobacco smoke is no less complicated as it enters the respiratory tract. Vaughan and Vaughan (1969) have drawn attention to the necessity for distinguishing between "deposition" and "retention" when studying the fate of inhaled aerosol particles. Nicotine arrives in the respiratory tract as part of an aerosol of semi-liquid, multi-component particles. Presumably some components of the aerosol may transfer rapidly from the particles to the blood stream depending on the chemical nature of the component itself. However, it is reasonable to believe that readily-absorbed substances such as nicotine may be taken up at variable rates depending on where the original aerosol lands.

It has been recognized for many years that studies of the absorption, actions and fate of nicotine must depend in large measure on highly-specific, sensitive means of detection of nicotine itself and its known and possible metabolites (McKennis et al., 1958, 1959, 1960 a & b). So far, the requirement for specificity has been met but the requirements for adequate sensitivity and range (in terms of metabolites) have not. McNiven et al. (1965) and Beckett and Triggs (1966) have reported selective extraction methods and protocols for detection by gas-liquid chromatography. However, 50 ng of nicotine begins to fall below the limits of detection. More recently, Isaac and Rand (1972) used an alkali flame ionization detector with gas-liquid chromatography and extended sensitivity to about 3 ng. Using the latter method, one can readily detect nicotine in blood immediately after smoking but levels become undetectable in a large proportion of subjects within two hours. Possibly of equal importance the method does not detect nicotine in the blood of heavy smokers after overnight abstinence. Of great practical importance, the extraction and detection methods are tedious and do not allow for the processing of large numbers of samples.

In contrast to the state of the art of measurement of nicotine, pharmacologic studies of the actions of nicotine have been carried out with rigor since the turn of the century. Even so, the situation described by Comroe in 1960 still exists: many physicians are familiar only with the effects of relatively large doses of nicotine applied locally or given intravenously. The questions remain, what does nicotine do in the doses inhaled in tobacco smoke, what are its usual metabolites and what concentrations are achieved in body fluids other than urine?

The recent successes in the development of radioimmunoassay of drugs, steroids and polypeptide hormones of body fluids in our laboratories (we now have assays for phenobarbital and morphine) indicates the feasibility of developing similar assays for nicotine and its major metabolites, cotinine, hydroxycotinine and desmethylcotinine (McKennis et al., 1958, 1959, 1960 a & b). In principal, we should be able to achieve sensitivities within the picagram range.

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We propose to develop these assays as a first step toward answering the question, what concentrations of nicotine and its metabolites are achieved in body fluids when nicotine is administered as a component of inhaled tobacco smoke? As indicated by results of preliminary studies, the assays can be converted to solid-phase protocols and can be automated as described in this proposed project. As suitable assay protocols are developed, we plan to make the protocols and the relevant antibodies available to all interested investigators.

Specific Aims

1. Develop specific conjugates of nicotine and its metabolites suitable for producing antibodies.

2. Evaluate the antibodies in terms of their specificities.

3. Work out "wet" chemistry protocols for the relevant radioimmunoassays.

4. Examine the suitability of the protocols for use in solid-phase.

5. Field test the final assay protocols.

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METHODS OF PROCEDURE

1. Conjugation of Nicotine and Related Substances to Protein

The chemical structures of nicotine and its metabolites are shown in Fig. 1. Since cotinine, hydroxycotinine and desmethylcotinine are structurally similar, it is highly desirable to produce antisera having no serious cross-reactions.

In the case of nicotine, we propose to couple via position-6 of the pyridine ring. Alternative procedures are shown in Fig. 2: firstly, 6-aminonicotine will be obtained by reacting sodium azide with nicotine. The rpoduct will be reacted with succinic anhydride to form its hemisuccinamide. The latter product can be coupled to bovine serum albumin (BSA) or to bovine gamma globulin (BGG) using carbodiimide.

Alternatively, 6-aminonicotine will be hydrolyzed to 6-hydroxynicotine (Chichibabin and Burkholtz, 1968), which in turn can be converted to 6-(4-aminobutoxy)-nicotine by reaction with N-(4-bromobutyl)-phthaliimide and subsequent acid hydrolysis. Again, coupling can be effected with carbodiimide.

We anticipate that by coupling via the pyridine ring, antibodies to nicotine and its major metabolites can be obtained with highly specific reactivities. Should this not be the case, coupling reactions applicable to the pyrolidine ring will be used. In the latter reactions, we will start with nornicotine reacted with 4-brombutynitrile, 4-bromocrotonate or succinic anhydride followed by hydrolysis. The products are amenable to condensation to a carrier such as thyroblobulin using carbodiimide.

2. Production and Evaluation of Antibodies

As a routine, each conjugate will be injected into fifteen rabbits. We plan to use 1 mg of hapten-protein conjugate emulsified in 1 ml of 50% complete Freund's adjuvant in saline per injection per rabbit. Weekly injections will be made through the first month and then biweekly injections during the second month. The rabbits thereafter will receive monthly injections. In our experience, three to six months are required for good antisera.

Titrations will be performed using serial dilutions. Affinity constants will be determined in the usual manner. Cross-reactivities will be determined using nicotine and its above-listed metabolites.

3. Assay Protocols and Sample Preparation

The proposed procedure for preparing samples is shown in Fig. 3. Should all of the antisera have serious cross-reactivity problems, a further separation will be effected using thin-layer chromatography.

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The nicotine levels in the blood of smokers has been reported to be 0.5-60 ng/ml of plasma (Isaac and Rand, 1972), depending on the smoking habits of each individual and the time elapsed after smoking. Since the efficiency of ether extraction of nicotine is greater than 90% (Isaac and Rand, 1969, 1972; Beckett and Triggs, 1966), the nicotine of the ether extract should be well above the anticipated minimum detectable level (20-30 pg/ml).

The addition of NaOH to plasma assures that nicotine and most of its metabolites will occur as free bases (nicotine: $pK_{a1} = 3$, $pK_{a2} = 8$ according to McKennis, 1960a). The possible exception is cotinine.

Twenty plasma samples, each from ten different plasma pools, will be prepared to contain serial concentrations of nicotine to be tested along with standard nicotine solutions to determine a) percent recoveries and b) the precision of the assays.

A Latin square design will be used as a preliminary evaluation of the radioimmunoassays.

e.g.

	Time			
Test	I	II	III	IV
1	B	C	A	D
2	C	A	D	B
3	D	B	C	A
4	A	D	B	C

Fifty plasma samples with four dose levels A, B, C and D will be given to four different researchers 1, 2, 3 and 4 at different times I, II, III and IV for the assay of nicotine and then its metabolites. The researchers will not know the dose levels with which they are working but will be given a series of nicotine standard solutions.

Theoretical considerations. The principle of radioimmunoassay is based on the competition of labelled and unlabelled antigens for specific antibody binding sites (Yalow and Berson, 1967; Odell and Daughaday, 1971).

In principle, the sensitivity of a radioimmunoassay can be evaluated from the following equation (See Appendix I-A):

$$\Delta C_H = (-\Delta b/b) \left[(C_H^* + C_H)(1-b)^2 + 1/k \right] / (1-b)^2 \quad (0 \leq b \leq 1) \quad (1)$$

in which K, C_H^*, C_H, and b are respectively the affinity constant of the antiserum, the concentrations of labelled and unlabelled haptens, and the fraction of the labelled hapten bound to the antibody.

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At minimum unlabelled hapten concentration, Eq. (1) becomes

$$\Delta C_H \doteq (-\, b/b) \left[C_H^* (1-b)^2 + 1/k \right] \;/\; (1-b)^2 \quad (0 \leq b \leq 1) \qquad (2)$$

This means that the minimum detectable hapten C_H is determined by the affinity constant of the antiserum, K, the concentration of the labelled hapten C_H^* used, and the change in bound hapten, Δb, due to the addition of C_H in the dilution of antiserum is such to give a fraction of bound hapten b.

Furthermore, if the affinity constant of the antiserum is sufficiently high to meet the criterion

$$1/K \ll C_H^* (1-b)^2 \qquad (0 \leq b \leq 1) \qquad (3)$$

Eq. (2) would become

$$C_H = C_H^* (-\Delta b/b) \qquad (0 \leq b \leq 1) \qquad (4)$$

The labelled nicotine available from commercial source at the present time has a specificity of 100-250 mc/m mole, equivalent to $22.2\text{-}55.5 \times 10^{10}$ DPM/m mole. Therefore, at least 650 pg of labelled nicotine (C_H^*) would be needed to get at least 1000 cpm in tests, if a 50% counting efficiency is assumed for counter.

The change in percentage bound, ($\Delta b/b \times 100$) could be considered to be significant only if the Δb is at least twice the standard deviation of b. That is, ($\Delta b/b \times 100$) has to be at least twice the coefficient of variation of the measurement of b. The minimum detectable nicotine estimated from Eq. (4) would be $C_H = 650 \times 0.04 = 26$ pg, if the coefficient of variation is 2%.

Thus, the sensitivity of the radioimmunoassay of nicotine will be estimated at about 30 pg (95% confidence), if the specific activity of labelled nicotine is higher than 250 mc/mmole and the affinity constants of the antisera meet the criteria (3). The latter requires the affinity constant $K \gg 10^8\ M^{-1}$.

The affinity constant of an antiserum at about $10^{10} M^{-1}$ seems to be common in antisera for steroids (Odell and Daughaday, 1971). The antisera against barbiturates and morphine also show affinity constants at the same order of magnitude. This indicates that the affinity constants of nicotine antisera may be also in this range.

A higher sensitivity could be achieved if labelled nicotine with a higher specific activity were available. On the other hand if the affinity constant of the antisera for nicotine could not meet the criteria (3), the sensitivity of this radioimmunoassay would be expected to be lower.

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4. Solid-Phase Protocols

Recently means have been found for immobilizing biological materials to insoluble carriers. In the case of antibodies, immobilization by covalent bonding can be carried out without affecting specific binding capacities (Messing, 1970; Weetall, 1969, 1970 a & b). On the basis of our preliminary experience with solid phase radioimmunoassays of thyroid stimulating hormone and progesterone, it appears that solid-phase antibodies can be used to reduce apparent antigen-antibody equilibrium times and therefore elapsed assay times by as much as seventy-fold.

Methods of insolubilization. Our experience so far has been with inorganic supports, in particular with silanized controlled pore glass (Corning Glass Works). We prefer inorganic supports because they are reusable, resistant to microbial contamination, are of high specific activity (and therefore are readily removed from suspension by centrifugation) and have sufficient intrinsic strength for use in columns developed under pressure. The major organic alternative, Agarose, is inferior in comparison with each of these points. Furthermore, in view of the large number of substituted silane reagents available, it is our opinion that a wider variety of derivatizing reactions can be used with silanized inorganic supports than with dextran gels or other organic polymers. There is the further point that inorganic carriers, once sized, do not fracture readily and therefore do not require repeated size characterizations.

With existing inorganic supports and silane reagents, it is possible to bind antibodies using acid chlorides, carbodiimide, diazo-reagents and isothiocyanate.

Antisera to nicotine and its metabolites will be coupled to controlled pore glass (mesh size 120/200, mean pore diameter 500 A, pore vol. 0.84 ml/g) silanized with γ-aminopropyl-triethoxysilane to produce an alkylamine glass. The alkylamine glass can be used directly or can be converted to an arylamine glass by refluxing with triethylamine and p-nitrobenzoylchloride. Glass beads of the dimensions described above are heavy enough to be removed by brief centrifugation yet are fine enough to resist rapid sedimentation by unit gravity. These properties give excellent control over the exposure of binding-protein to antigen and discrete termination of exposure. Assay protocols will be based on the specific wet chemistry protocols described previously.

5. Field Tests

In order to establish reproducible routine procedures for the radioimmunoassays of nicotine and its major metabolites, animal experiments will be performed as follows:

a. Sixty male and sixty female rabbits will be divided into 12 groups as shown in the following table:

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Source: https://www.industrydocuments.ucsf.edu/docs/xxyl0000

Dose (ug)	Male Young	Male Adult	Female Young	Female Adult
1	10	10	10	10
2	10	10	10	10
4	10	10	10	10

Rabbits 3-4 weeks and 4-5 weeks of age will be grouped as "young" and "adult." In these experiments, nicotine will be administered by a single intravenous injection. These dose levels correspond to the blood nicotine levels after smoking 1/2 to 2 cigarettes. Seven 4 ml samples of blood will be taken from each rabbit at 2,4,8,16,32,64 and 128 minutes after the injection and will be tested for nicotine by radioimmunoassay and by gas-liquid chromatography.

b. In the second test, 160 male and 160 female rabbits will be divided into 16 groups as shown below:

Dose (ug)	Male Young	Male Adult	Female Young	Female Adult
2	20	20	20	20
4	20	20	20	20
6	20	20	20	20
8	20	20	20	20

These doses correspond to the nicotine levels achieved in blood after 1 to 4 cigarettes. Nicotine will again be administered by a single intravenous injection once every two weeks. Blood samples will be taken twice, once at one hour and the other at one week after injection. In order to handle these large numbers of animals, each group will be divided into five subgroups, such that 20 rabbits can be handled 4 at a time in five week days. The blood samples will be tested by radioimmunoassay and gas-liquid chromatography.

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Source: https://www.industrydocuments.ucsf.edu/docs/rrylfdd1

SIGNIFICANCE

This project is aimed at the development of radioimmunoassays of nicotine, cotinine, hydroxycotinine and desmethylcotinine to answer the question, what levels of these compounds are achieved in body fluids as a consequence of smoking? Through the development of these assays, we then plan to make the protocols and antisera available to all interested investigators to allow reexamination of the question, what are the actions of nicotine and its metabolites in man in the doses inhaled in tobacco smoke?

Existing assay methodologies are too insensitive to detect nicotine in the concentrations likely to occur in blood one or two hours after smoking. In addition, virtually nothing is known of the immediate fate of nicotine or of the concentrations of its metabolites achieved in body fluids other than urine. The metabolites, if they occur in blood, may well be of interest not only in terms of carcinogenesis (e.g. aromatic amines and cancer of the bladder) but also in terms of nicotinic effects on cardiac conduction, sympathetic and parasympathetic synaptic events, tracheal epithelial ciliary motion and neuromuscular transmission. However, we believe that investigations of these possible actions should await a clear understanding of the concentration of nicotine and its metabolites likely to arrive at possible target sites. Clearly, the magnitude of possible physiologic and pharmacologic experiments exceeds our expertise and available manpower. Therefore the emphasis of this project is on developing and making available the materials and protocols necessary for highly sensitive, specific and simple assays.

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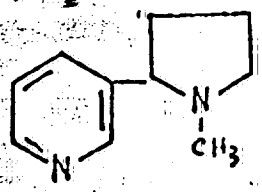
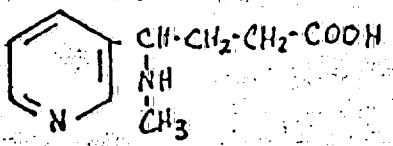
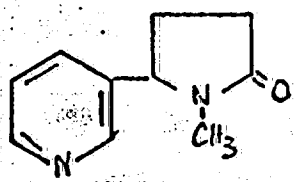


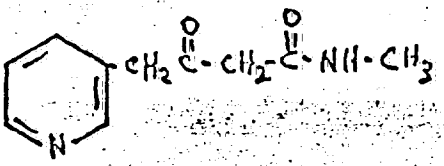
(-)-nicotine



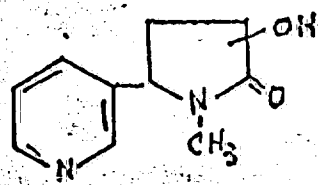
(+)- α-3-(pyridyl)-α-methylaminobutyric acid



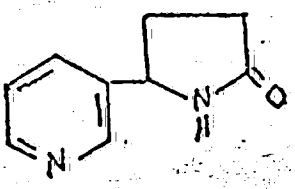
(-)-cotinine



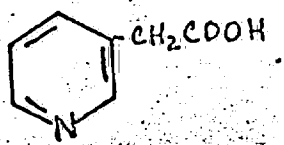
α-(3-pyridyl)-β-oxo-N-methylbutyramide



Hydroxycotinine



(-)-desmethylcotinine



3-pyridylacetic acid

Figure 1. Major Nicotine Metabolites

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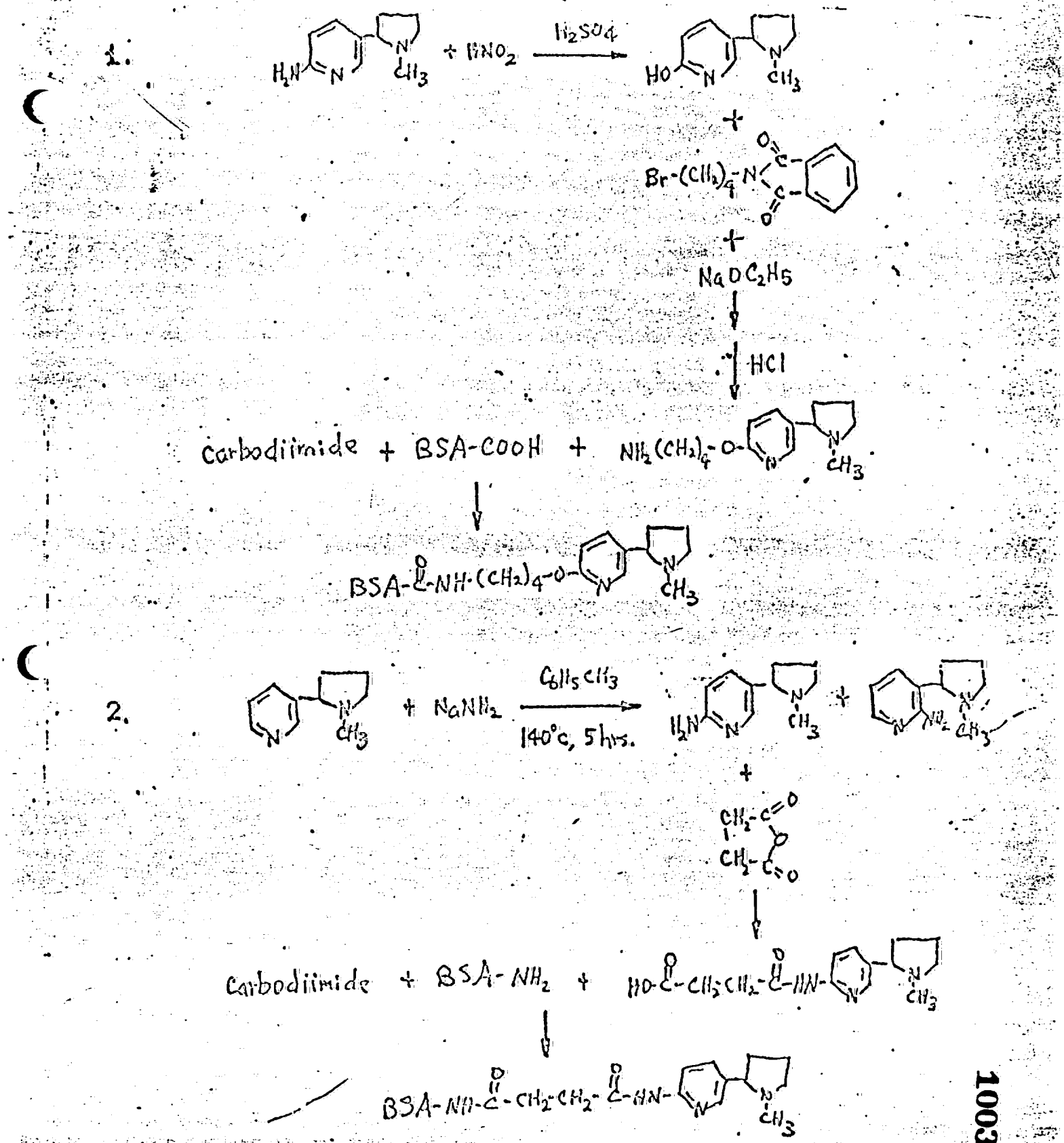

Figure 2. Synthesis and Conjugates of Nicotine to BSA

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Source: https://www.industrydocuments.ucsf.edu/docs/nyb0000

0.1 ml plasma (contains 0.05 to 6 ng nicotine)

+ 0.02 ml 5M NaOH

+ 3 x 0.5 ml ether extraction

This could be circumvented if the specificity of nicotine antiserum is high.

1.2 ml of ether extract (in a 8 x 75 mm tube)

evaporate to dryness

+ 0.1 ml of labeled nicotine - H^3 (x 1500 cpm) buffer solution

+ 0.1 ml of nicotine antiserum buffer solution

Incubate overnight at 4°C

+ 0.2 ml of saturated $(NH_4)_2SO_4$ solution

Centrifuge at 3500 rpm for 20 min. at 4°C

0.2 ml supernant in a counting vial

+ 10 ml scintillation fluid

Shake for 10 minutes

Count free nicotine - H^3

Compare with standard curve

Calculate nicotine content

Figure 3. Proposed procedure for determination of nicotine in blood by radioimmunoassay.

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Source: https://www.industrydocuments.ucsf.edu/docs/nlyl0000

The GLC method reported by Isaac and Rand (1972) will be used in this study.

2.5 ml plasma

+3 ml of 15 ug/ml modaline soln

+1 ml of 5 M NaOH

3 x 5 ml ether extraction

about 11 ml ether extract

evaporated on 42°C water bath

10 ul ether extract

2 ul ether extract injected to GLC

Calculate the nicotine content

Figure 4. Detection of Nicotine by GLC

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Source: https://www.industrydocuments.ucsf.edu/docs/nryl0000

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Facilities

1. Space. The space will be comprised of 5000 sq. ft. with facilities for high and low levels of radioactivity, counting room, labelling room, electron microscope room, peptide synthesis and amino acid analyzer, general lab, pump room, organic lab, cold room, data room, conference room, and offices. This space will be shared with Doctors J.W. Ryan and Una Smith, Senior Scientists of the Papanicolaou Cancer Research Institute. Rabbits are boarded in the Central University facility.

2. Major Items of Equipment

Philips EM 100 electron microscope
LKB knifemaker
Durst enlarger and ancillary preparative and photographic equipment
Zeiss binocular microscope with camera and phase contrast attachments
Gilson and LKB fraction collectors
Craig-Post 200-transfer countercurrent distributor
Spinco 120C amino acid analyzer
Unilux II liquid scintillation counter
Actigraph strip counter
Gilford-Beckman recording spectrophotometer
Buchi flash evaporator
Grass four-channel polygraph
Radiometer automatic titrator
Radiometer pH meters (2)
Mettler top pan balance
Sartorius analytical balance
Apparatus for cleavage with anhydrous HF
Apparatus for paper and thin layer chromatography and electrophoresis
Pumps for infusion and respiration of small animals
Vacuum oven
Polarimeter
Circulating water baths and incubators
Apparatus for manual and automatic synthesis of polypeptides
Virtis lyophilizer
Two channel auto analyzers
Sowall RCB-2 centrifuge
Two respirators (one cat, one rabbit-cat)

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Appendix A - Derivation of Equation (1)

From the references (15-17), the equilibrium of hapten, antibody, and antibody-hapten system can be expressed as:

$$A + H^* \rightleftharpoons AH^* \qquad \text{(A-1)}$$

$$A + H \rightleftharpoons AH \qquad \text{(A-2)}$$

where A, H, H*, AH, and AH* are respectively antibody, unlabeled and labeled haptens, and unlabeled- and labeled-antibody complexes. The affinity constants (apparent equilibrium constants) for antibody would be

$$K_H^* = [AH^*] \,/\, [AH^*] \qquad \text{(A-3)}$$

$$K_H = [AH] \,/\, [A]\,[H] \qquad \text{(A-4)}$$

The mean the concentrations.

The material balances are:

$$C_A = Co/n = [A] + [AH^*] + [AH] \qquad \text{(A-5)}$$

$$C_H^* = [H^*] + [AH^*] \qquad \text{(A-6)}$$

$$C_H = [H] + [AH] \qquad \text{(A-7)}$$

where C_A, C_H, AND C_H are respectively the total concentrations of antibody labeled, and unlabeled haptens, and Co is the initial antibody concentration in antiserum prior to an n-fold dilution. Eqs. (A-3) and (A-4) may be rewritten as:

$$[A] = [AH^*] \,/\, K_H^* \,.\, [H^*] \qquad \text{(A-8)}$$

and

$$[AH] \,/\, K_H \,.\, [A] \qquad \text{(A-9)}$$

From Eqs. (A-7) and (A-9), one can obtain:

$$AH = \frac{C_H}{1 + K_H [A]} \qquad \text{(A-10)}$$

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Substitution Eq. (A-8) to Eq. (A-10) gives:

$$[AH] = \frac{C_H}{1 + K_H^* \; [H^*] \; / \; [AH^*]} \qquad \text{(A-11)}$$

If the antibody does not discriminate against either labeled hapten or unlabeled hapten, K_H^* would be equal to K_H.

Let $K = K_H^* = K_H$, $b = [AH^*] \; / \; C_H$, and $f = [H^*] \; / \; C_H^*$, then from eqa. (A-8), (A-11), and (A-5) one obtains:

$$C_A = b/Kf + \; C_H^* + C_H) \; b \qquad (0 \leq b \leq 1) \qquad \text{(A-12)}$$

$$C_A = b/K \; (1-b) + (C_H^* + C_H) \; b \qquad (0 \leq b \leq 1) \qquad \text{(A-13)}$$

Differentiation of Eq. (A-13) results:

$$dC_H/db - \left[1/K + (C_H^* + C_H) \; (1-b)^2\right] \; /b \; (1-b)^2 \qquad \text{(A-14)}$$

This can be rewritten as:

$$dC_H = (-\Delta b/b) \; \left[1/K + (C_H^* + C_H) \; (1-b)^2\right] \; /(1-b)^2$$

$$(0 \leq b \leq 1) \qquad \text{(A-15)}$$

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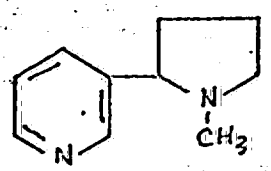
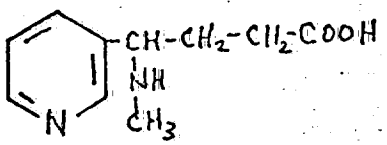
Appendix A-2

NICOTINE ALBUMIN CONJUGATE

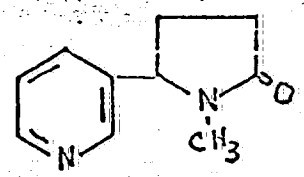
The major nicotine metabolites have been identified as (+) -α-3-(pyridyl) -α-methylaminobutyric acid. (-)-cotinine α-(3-pyridyl) B-oxo-N-methylbutyramide, hydroxycotinine, (-) desmethylcotinine and 3-pyridylacetic acid with the following structure:



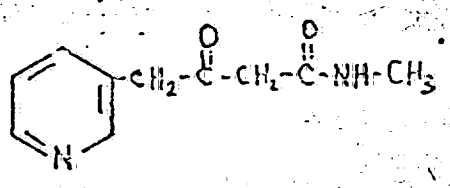
(-)-nicotine



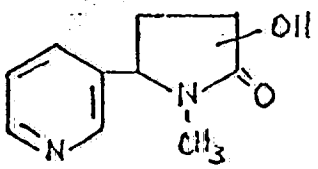
(+)-α-3-(pyridyl)-α-methylaminobutyric acid



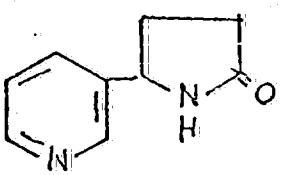
(-)-cotinine



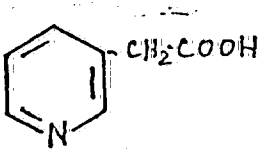
α-(3-pyridyl)-β-oxo-N-methylbutyramide



Hydroxycotinine



(-)-desmethylcotinine



3-pyridylacetic acid

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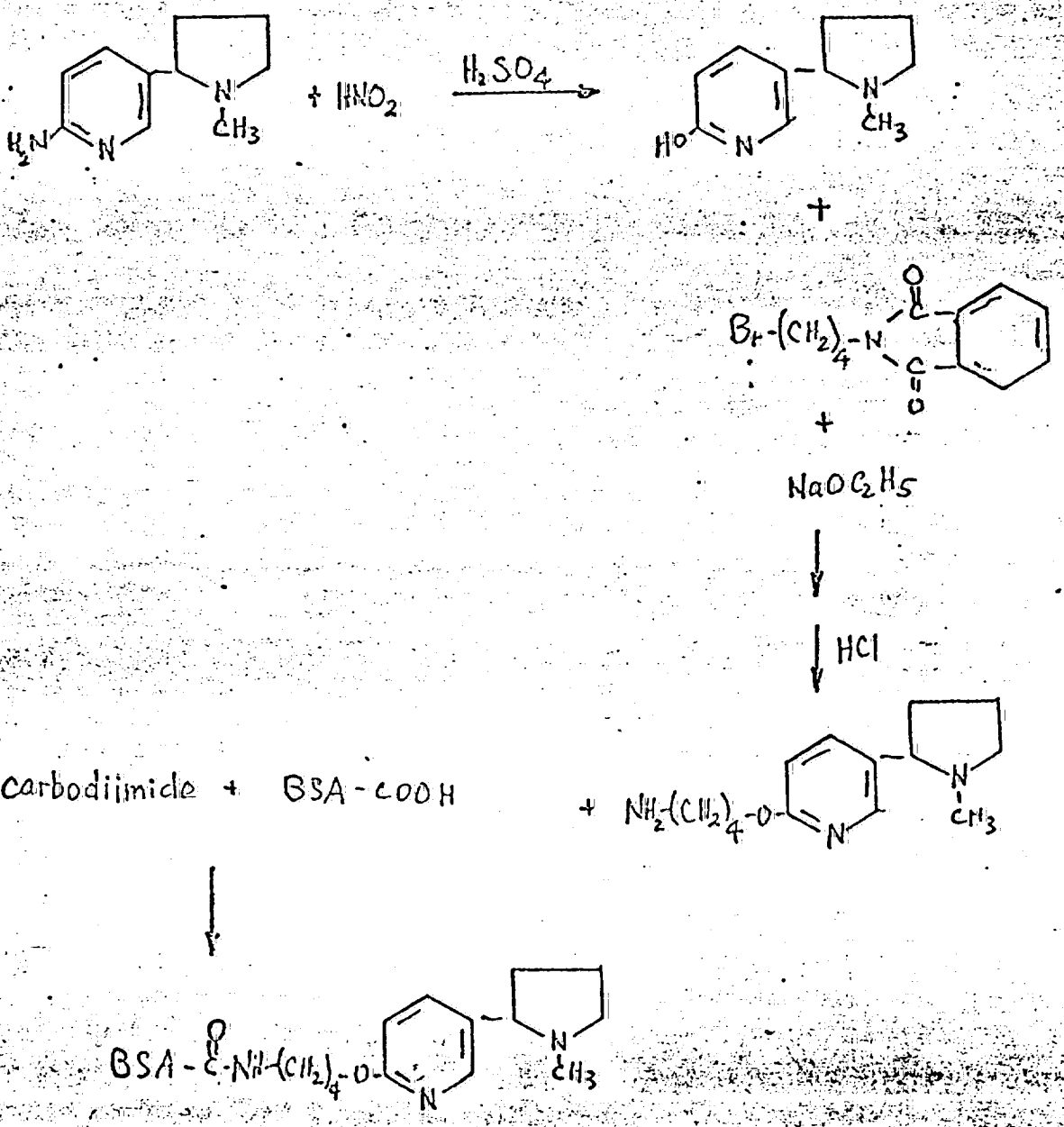
We have found that some of the compounds consisting of both amino and acid group in the same molecule can form a strong intramolecular hydrogen bond and hence interfer with the hapten albumin conjugation. Alternatively we propose to synthesize 6-hydroxynicotine by the action of HNO_2 in the presence of H_2SO_4. The sodium salt of 6-hydroxynicotine can then be reacted with N-(4-bromobutyl)-phthalimide; and the free amine formed upon hydrolysis.



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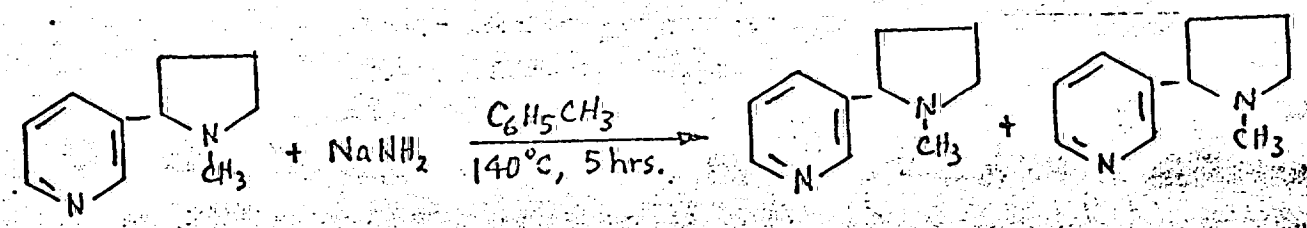
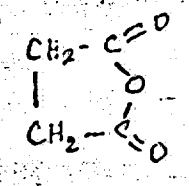
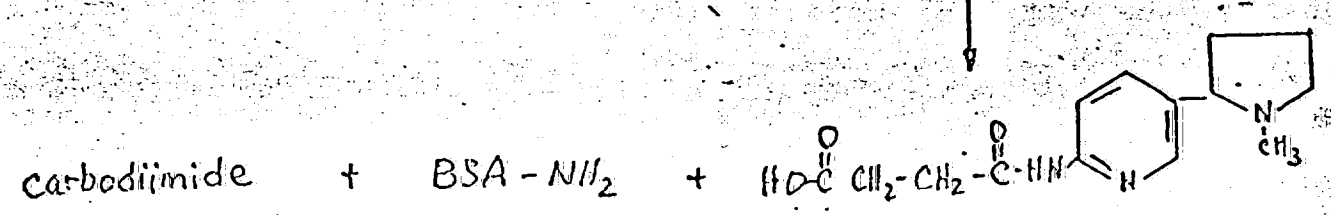
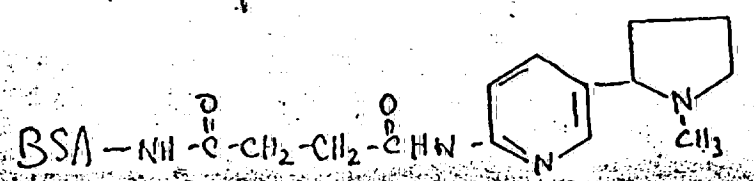
It is well known that the antigenic sensitivity and specificity is predominately directed toward the distal portion of the hapten. It seems reasonable to conjugate the nicotine to albumin through either five or six position of the pyridyl ring in order to distinguish nicotine from its metabolites.

Nicotine is first reacted with sodium amide to produce 6-amino nicotine. The product subsequently reacted with succinic anhydride and then conjugated to albumin.









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Biographical Sketch

Albert Castro Principal Investigator

b. November 15, 1933, San Salvador, El Salvador Central America — Permanent resident, U.S.

Major research interest: Hormonal regulation of blood pressure and its pathological disturbance in hypertension

Education:

Institution and Location	Degree	Year Conferred	Scientific Field
University of Houston, Houston, Texas	B.S.	1958	Biology
Baylor University, Jeff Davis Hosp., Houston, Texas	M.T.	1958	
University of El Salvador, El Salvador, C.A.	Ph.D.	1962	Biological Chemistry

Honors:

PHS Senior Postdoctoral Fellowship, 1965-1969.
Award and Member, ATENEO, Academy of Science of El Salvador
General Relator, 3rd International Congress of Dental Education (127 universities) Mexico, 1964.
Northwest Pediatric Research

Professional experience:

1969---- Assistant Professor of Pediatrics and Co-Director, Pediatric Renal-Metabolic Laboratory, University of Oregon Medical School, Portland, Oregon.

1968-70 Senior Research Fellow, Diabetes and Metabolism. University of Oregon Medical School, Portland, Oregon.

1966-68 Postdoctoral Research Fellowship, Diabetes and Hypertension. University of Oregon Medical School, Portland, Oregon.

1967 Course in Radioisotopes in Medicine, University of Oregon Medical School, Portland, Oregon, under Dr. E. Hine, August, 1967.

1963 Course in Gas and Thin Layer Chromatography, Department of Chemistry, University of El Salvador. Under Professor J. Kannan.

1958-60 Assistant Professor of Microbiology and Biochemistry, Dental and Chemistry Schools, University of El Salvador, C.A.

1960-63 Associate Professor of Biochemistry and Microbiology, Dental and Medical Schools, University of El Salvador, C.A.

1965-66 Professor and Head, Department of Basic Sciences, Dental School, University of El Salvador, C.A.

1965-66 Co-Director of Graduate Research, Faculty of Chemistry, University of El Salvador, C.A.

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10. Price, L., Stone, G., and Castro, A.: Perspectives in thyroid testing. Lab News, p. 12-23, March, 1971.
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